Evidence for Selective Effects of Chronic Hypertension on Cerebral Artery Vasodilatation to Protease-Activated Receptor-2 Activation

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Background and Purpose—Protease-activated receptor-2 (PAR-2) can be activated after proteolysis of the amino terminal of the receptor by trypsin or by synthetic peptides with a sequence corresponding to the endogenous tethered ligand exposed by trypsin (eg, SLIGRL-NH₂). PAR-2 mediates nitric oxide (NO)–dependent dilatation in cerebral arteries, but it is unknown whether PAR-2 function is altered in cardiovascular diseases. Since hypertension selectively impairs NO-mediated cerebral vasodilatation in response to acetylcholine and bradykinin, we sought to determine whether PAR-2–mediated vasodilatation is similarly adversely affected by this disease state.

Methods—We studied basilar artery responses in Wistar-Kyoto rats (WKY) (normotensive) and spontaneously hypertensive rats (SHR) in vivo (cranial window preparation) and in vitro (isolated arterial rings). The vasodilator effects of acetylcholine, sodium nitroprusside, and activators of PAR-2 and protease-activated receptor-1 (PAR-1) were compared in WKY versus SHR. Immunohistochemical localization of PAR-2 was also assessed in the basilar artery.

Results—Increases in basilar artery diameter in response to acetylcholine were 65% to 85% smaller in SHR versus WKY, whereas responses to sodium nitroprusside were not different. In contrast to acetylcholine, vasodilatation in vivo to SLIGRL-NH₂ was largely preserved in SHR, and SLIGRL-NH₂ was 3-fold more potent in causing vasorelaxation in SHR versus WKY in vitro. In both strains, responses to SLIGRL-NH₂ were abolished by N^G-nitro-L-arginine, an inhibitor of NO synthesis. Activators of PAR-1 had little or no effect on the rat basilar artery. PAR-2–like immunoreactivity was observed in both the endothelial and smooth muscle cells of the basilar artery in both strains of rat.

Conclusions—These data indicate that NO-mediated vasodilatation to PAR-2 activation is selectively preserved or augmented in SHR and may suggest protective roles for PAR-2 in the cerebral circulation during chronic hypertension. (Stroke. 1999;30:1933-1941.)

Key Words: basilar artery n endothelium n nitric oxide n thrombin

Protease-activated receptors (PARs) include the thrombin receptors PAR-1 and PAR-3, the trypsin receptor PAR-2, and PAR-4, which can be activated by both trypsin and thrombin. These novel G-protein–coupled receptors initiate cell signaling after site-specific proteolysis by thrombin or trypsin to unmask amino terminals that function as tethered ligands to self-activate each receptor. In addition, synthetic peptides corresponding to the tethered ligand sequences of PAR-1, PAR-2, and PAR-4 (SFFLRN, SLIGRL, and GYPGKF, respectively, for the mouse and rat receptors), but not that for the mouse PAR-3 (TFRGAP), cause activation of these receptors. PARs are expressed by several cell types in blood vessels, including endothelial and smooth muscle cells. It is well established that, apart from platelet activation, thrombin and synthetic PAR-1 agonist peptides modulate the tone of isolated arteries, consistent with a possible role of PAR-1 in control of blood flow in vivo. Additionally, PAR-2 most likely contributes to regulation of vascular tone, since PAR-2 mRNA is present in highly vascularized tissues, and application of trypsin or synthetic PAR-2 agonist peptides causes endothelium-dependent relaxation of isolated peripheral arteries. Little, however, is known of the importance of PARs in regulating tone of cerebral arteries, although thrombin has been reported to cause endothelium-dependent relaxation and direct contraction of human and isolated basilar arteries. In addition, we recently reported that activation of PAR-2 elicits nitric oxide (NO)–dependent dilatation of rat cerebral arteries in vivo.

The effects of pathological changes to the cardiovascular system on expression and function of PARs in either cerebral or noncerebral blood vessels have not yet been evaluated, although the proinflammatory mediator tumor necrosis factor-α has been shown to upregulate PAR-2 mRNA in

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isolated endothelial cells. Chronic hypertension is a cardiovascular disease and, although not yet described as an inflammatory vascular state, it is known to predispose to stroke and cause alterations in the function of cerebral vascular smooth muscle and endothelial cells. We have shown that PAR-2 mediates powerful NO-dependent dilatation of the cerebral vasculature of normotensive rats in vivo. Therefore, since chronic hypertension results in endothelial dysfunction and thus loss of responsiveness to acetylcholine, the aim of this study was to determine whether changes to PAR-2–mediated cerebral vasodilatation occur after the development of chronic hypertension. Our data indicate that NO-dependent cerebral vasodilator responses to activation of PAR-2 are, in contrast to those to acetylcholine, selectively preserved or augmented during chronic hypertension. We also found immunoreactive PAR-2 in both endothelial and vascular smooth muscle cells of the basilar artery from both strains of rat even though no direct contraction was observed to PAR-2 activation after inhibition of NO synthase.

**Materials and Methods**

Procedures used in these experiments were approved by the University of Melbourne Animal Experimentation Ethics Committee. Experiments were performed in 6- to 11-month-old male Wistar-Kyoto rats (WKY) (n=22) and spontaneously hypertensive rats (SHR) (n=21).

**In Vivo Experimental Protocol**

WKY (n=10) and SHR (n=9) were anesthetized with pentobarbital sodium (50 mg/kg IP) supplemented at 10 to 20 mg · kg⁻¹ · h⁻¹ IV. A tracheostomy was performed for mechanical ventilation with room air and supplemental oxygen. A femoral artery catheter was used to measure arterial pressure and to obtain arterial blood, and a femoral vein was cannulated for injection of supplemental anesthetic. Arterial blood gases and pH were maintained at normal levels for the duration of the experiment (pH=7.39±0.01; PCO₂=36±1 mm Hg; PO₂=160±11 mm Hg). Body temperature was monitored continuously with a rectal probe and was maintained at 37°C to 38°C with a heating pad. The rat was then placed in a head holder in a supine position. The larynx and esophagus were retracted rostrally and laterally, and the musculature covering the basioccipital bone was removed. A craniotomy was then performed over the ventral brain stem, and the dura was incised to expose the basilar artery. The cranial window was then superfused with artificial cerebrospinal fluid (composition [mmol/L]: Na⁺154.6, Cl⁻139.7, HCO₃⁻26.4, K⁺2.95, Ca²⁺1.71, Mg²⁺0.65, and d-glucose 5.69) at 37°C to 38°C. When sampled from the cranial window, cerebrospinal fluid gases and pH were as follows: PCO₂=38±1 mm Hg, PO₂=111±4 mm Hg, and pH=7.34±0.01. Diameter of the basilar artery was measured with a microscope equipped with a television camera coupled to a video monitor and was continuously measured with the use of a computer-based tracking program (Diamtrak; Montech). The basilar artery was allowed to equilibrate for 30 minutes after the preparation of the cranial window before vasodilator responses were obtained to topical application of the following agents: (1) acetylcholine (10⁻⁶ to 10⁻⁸ mol/L), which causes dilation of the basilar artery by releasing endothelium-derived NO24–26; (2) sodium nitroprusside (10⁻⁶ to 10⁻⁸ mol/L), a NO-donating endothelium-independent agonist; (3) SLIGRL-NH₂ (10⁻⁶ to 10⁻⁸ mol/L), the latent sequence of the PAR-2 tethered ligand peptide, which causes NO-dependent dilatation of the basilar artery27; (4) trypsin (0.01 to 1 U/mL), which enzymically activates PAR-2; (5) SFLLRN-NH₂ (10⁻⁶ to 10⁻⁸ mol/L), the human sequence of the PAR-1 tethered ligand peptide which is equipotent at rat PAR-125; and (6) thrombin (0.01 to 1 U/mL), which enzymically activates PAR-1. Drugs, diluted in artificial cerebrospinal fluid, were then superfused over the cranial window in cumulatively increasing concentrations. The diameter of the basilar artery was recorded under basal conditions and during application of each concentration of agonist. Vessel diameter returned to control levels within a few minutes of removing each agonist, and then an additional 15-minute recovery period was allowed before application of another agonist. The sequence of application of the agonists was randomized. Responses of the basilar artery were stable within 3 to 5 minutes of commencing application of each agonist.

The reproducibility of responses to SLIGRL-NH₂ was determined in some rats. Thus, we recorded dilatations of the basilar artery to SLIGRL-NH₂ (10⁻⁶ to 10⁻⁸ mol/L) using the experimental protocol described above, followed 60 minutes later by a second application of SLIGRL-NH₂.

Finally, the possibility that dilator responses of the basilar artery to SLIGRL-NH₂ were mediated by NO production was examined in WKY (n=4) and SHR (n=4) in which responses to SLIGRL-NH₂ were recorded during the experimental protocol described above. Then, after 40 minutes of recovery and an additional 20-minute exposure to the NO synthase inhibitor N⁶-nitro-L-arginine (L-NNA; 10⁻⁴ mol/L), responses to repeated applications of SLIGRL-NH₂ were measured in the continued presence of L-NNA.

**In Vitro Experimental Protocol**

Animals (WKY, n=9; SHR, n=9) were killed with an overdose of pentobarbital sodium (40 mg IP), and the brain was rapidly removed and placed in ice-cold, carbogenated (95% O₂, 5% CO₂) Krebs’ solution (composition [mmol/L]: Na⁺144, Cl⁻128.7, HCO₃⁻25, K⁺5.9, Ca²⁺2.5, Mg²⁺1.2, H₂PO₄⁻1.2, SO₄²⁻1.2, and glucose 11, pH 7.4). The basilar artery was carefully dissected free from surrounding tissues and divided into 2-mm-long segments. These segments were mounted on 40-µm stainless steel wires in a Multivane-style small-vessel myograph (J.P. Trading) in Krebs’ solution and allowed to equilibrate at 37°C for 30 minutes. After equilibration, the preparations were stretched to a passive tension of 5 mN, which we found to be optimal in preliminary experiments, and allowed to further equilibrate for 30 minutes. The maximum contractile capacity of each preparation (Fmax) was then estimated by replacing the normal Krebs’ solution with an isotonic high-potassium Krebs’ solution (120 mmol/L KCl, substituted for NaCl). Thirty minutes after washout of the high-potassium solution, the preparations were contracted to ~50% Fmax with titrated concentrations of 5-hydroxytryptamine (5-HT) (10⁻⁶ to 10⁻⁷ mol/L), and relaxations to cumulatively increasing concentrations of PAR agonists or acetylcholine were obtained. The maximum relaxation of the tissue was determined after the addition of the nitric oxide synthase inhibitor ISL, together with isobutylmethylxanthine (10⁻⁴ mol/L) after the maximum relaxation to the PAR activators or acetylcholine was obtained.

**Immunohistochemistry**

Animals (WKY, n=3; SHR, n=3) were killed as described above and perfused transcardially with PBS (4°C, pH 7.3) for 3 minutes followed by 4% paraformaldehyde in phosphate buffer (4°C, pH 7.3) for 5 minutes. The brain was removed and placed in the same fixative at 4°C for 16 hours before thorough washing in PBS. A portion of the ventral brain stem, containing the basilar artery, was then removed and embedded in paraffin. Microtome sections (3 µm) were deparaffinized and rehydrated before application of a rabbit antisera against the carboxy-terminal of PAR-2 (RAB 9717, 1:250 to 1:500; a kind gift of Professor Nigel Bunnett, University of California, San Francisco) for 36 hours in a humid chamber at room temperature. After they were washed in PBS, the sections were labeled with a biotinylated donkey anti-rabbit antisera (Amer sham) before further washing with PBS and application of a streptavidin-FITC complex (Amer sham). To check the specificity of the primary antisera, in some experiments sections were incubated with the PAR-2 antisera after preabsorption with the antigenic peptide sequence CSVKTSY. The sections were mounted in buffered glycerol (pH 8.6) and examined under epifluorescence with a Zeiss AxiosSkop microscope equipped with an FITC filter set. Photographs were taken on Kodak Ektachrome 160T color slide film.
at 320 ASA; digitized black and white figures were prepared on a personal computer with the use of Adobe Photoshop software.

**Drugs**

Acetylcholine chloride, isobutyl methylxanthine, L-NNA, 5-HT, and sodium nitroprusside were obtained from Sigma Chemical Co. Rat PAR-2 agonist peptide (SLIGRL-NH$_2$; molecular weight = 657) and PAR-1 agonist peptide (SFLLRN-NH$_2$; molecular weight = 748) were obtained from Auspep. Trypsin (bovine pancreas; molecular weight = 24 000) and a-thrombin (molecular weight = 33 580) were obtained from Worthington Biochemical Corp (Freehold, NJ). Pentobarbital sodium was obtained from Rhone Merieux. Aliquots of stock solutions of SLIGRL-NH$_2$ (10$^{-2}$ mol/L), SFLLRN-NH$_2$ (10$^{-2}$ mol/L), trypsin (1000 U/mL), and thrombin (1000 U/mL) were prepared in distilled H$_2$O and stored at −20°C. All subsequent dilutions of these drugs were made in distilled water (in vitro experiments) or saline (in vivo experiments).

**Statistical Analysis**

In vivo vascular responses are presented as percent change in diameter of the basilar artery. In vitro vascular relaxations are presented as decreases in the 5-HT–induced contraction caused by each agonist as a percentage of the maximum relaxation produced in response to 10$^{-6}$ mol/L sodium nitroprusside and 10$^{-4}$ mol/L isobutyl methylxanthine. Cumulative concentration–relaxation curves from each in vitro experiment were fitted to a sigmoidal logistic equation to derive EC$_{50}$ values (Prism, Graphpad). All data are expressed as mean±SE. Single comparisons were made with Student’s paired or unpaired t tests, as appropriate. Differences in variation between groups were compared with an F test. If a significant difference in variation was observed, the difference between the means was assessed by a t test with Welch’s correction. A value of P<0.05 was considered significant.

**Results**

**In Vivo Experiments**

Mean arterial blood pressure averaged 95±4 mm Hg in WKY and 180±6 mm Hg in SHR (P<0.05). Arterial pressure was not affected by application of vasodilators in the cranial window (data not shown). Basilar artery baseline diameter averaged 259±7 μm in WKY and 216±4 μm in SHR (P<0.05).

**Responses to Acetylcholine and Sodium Nitroprusside**

Concentration-dependent vasodilatation to acetylcholine in the basilar artery, which reached a maximum increase in diameter of ≈25% at 10$^{-4}$ mol/L in WKY, was markedly impaired in SHR (Figure 1a). In contrast, sodium nitroprusside caused similar concentration-dependent dilatation of the basilar artery in WKY and SHR, to a maximum of ≈60% increase in diameter at 10$^{-5}$ mol/L in both strains (Figure 2). These findings confirm previous studies$^{26,29}$ that showed similar selective impairment of basilar artery responses to acetylcholine in vivo in chronic hypertension.

**Responses to the PAR-1 and PAR-2 Agonist Peptides SFLLRN-NH$_2$ and SLIGRL-NH$_2$, Respectively**

SLIGRL-NH$_2$ caused concentration-dependent basilar artery vasodilatation in WKY to a maximum of ≈50% increase in diameter at 10$^{-5}$ mol/L (Figure 1b), confirming our previous finding in normotensive Sprague-Dawley rats that PAR-2 mediates profound cerebral vasodilatation.$^{21}$ While the vasodilator response to the lowest concentration (10$^{-6}$ mol/L) of SLIGRL-NH$_2$ was significantly reduced in SHR (5±2%) compared with that in WKY (10±2%; P<0.05), the responses to higher concentrations (3×10$^{-6}$ and 10$^{-5}$ mol/L) of SLIGRL-NH$_2$ in SHR were not significantly different from those in WKY (Figure 1b).

In contrast to SLIGRL-NH$_2$, the PAR-1 activating peptide SFLLRN-NH$_2$ had no effect on basilar artery diameter in WKY or SHR (Figure 1c), indicating that PAR-1 is not functional in regulating rat basilar artery tone under normal conditions and that chronic hypertension does not affect PAR-1 function in this artery.

**Effect of L-NNA on Vasodilator Responses to SLIGRL-NH$_2$**

The dilator responses to SLIGRL-NH$_2$ in the basilar artery were reproducible with time in both WKY (data not shown)
and SHR (Figure 3a). In separate experiments, L-NNA (10^{-4} \text{ mol/L}) decreased basilar artery diameter in both strains (WKY, control = 269 ± 5 \mu\text{m} versus L-NNA–treated = 204 ± 15 \mu\text{m}, n = 4, P < 0.05; SHR, control = 210 ± 4 \mu\text{m} versus L-NNA–treated = 183 ± 7 \mu\text{m}, n = 4, P < 0.05). When a second application of SLIGRL-NH\textsubscript{2} was given in the presence of L-NNA, vasodilator responses were markedly attenuated in both WKY and SHR (P < 0.05). For example, in WKY under control conditions, SLIGRL-NH\textsubscript{2} (10^{-6}, 3 \times 10^{-6}, and 10^{-5} \text{ mol/L}) increased basilar artery diameter by 12 ± 4%, 30 ± 3%, and 43 ± 2%, respectively. In contrast, in the presence of L-NNA, increases in diameter produced by SLIGRL-NH\textsubscript{2} in WKY were 0 ± 0%, 4 ± 3%, and 13 ± 3%, respectively (n = 4; all values P < 0.05 versus control). Similar data from SHR presented in Figure 3b show inhibition of responses to SLIGRL-NH\textsubscript{2} by L-NNA. These findings suggest that NO production mediated cerebral vasodilator responses to PAR-2 in both WKY and SHR.

**Responses to Thrombin and Trypsin**

Both thrombin and trypsin had little or no effect on basilar artery diameter in both WKY and SHR (Figure 4).

**In Vitro Experiments**

**Responses to SLIGRL-NH\textsubscript{2} and SFLLRN-NH\textsubscript{2}**

Although the maximal relaxations of isolated basilar arteries to SLIGRL-NH\textsubscript{2} were not significantly different between the 2 strains of rat (R\textsubscript{max}; WKY, 78.4 ± 4.8%; SHR, 89.8 ± 0.7%; P = 0.064), the potency of SLIGRL-NH\textsubscript{2} was significantly ≈3-fold greater in preparations from SHR rats (pEC\textsubscript{50} [−log mol/L]; WKY, 5.97 ± 0.18; SHR, 6.47 ± 0.05; P < 0.05; Figure 5a). Notably, there was also significantly less variation in the pEC\textsubscript{50} of SLIGRL-NH\textsubscript{2} in preparations from SHR rats (F test, P < 0.05; Figure 5a). In contrast to SLIGRL-NH\textsubscript{2}, high concentrations (10^{-5} \text{ mol/L}) of SFLLRN-NH\textsubscript{2} only caused relatively small relaxations (R\textsubscript{max}; WKY, 17.5 ± 8.3%; n = 4; SHR, 24.6 ± 7.3%, n = 4).

**Responses to Trypsin and Thrombin**

Thrombin (0.01 to 1 U/mL) had no effect on preparations from either strain (WKY, n = 4; SHR, n = 4; data not shown). Trypsin, however, caused similar concentration-dependent relaxation in preparations from both strains (R\textsubscript{max}; WKY, 82.6 ± 4.4%; SHR, 87.6 ± 2.6%; pEC\textsubscript{50} [−log U/mL]; WKY, 1.8 ± 0.1; SHR, 1.2 ± 0.3; Figure 5b).

**Responses to Acetylcholine**

Acetylcholine caused equipotent relaxations in isolated basilar artery preparations from both strains (pEC\textsubscript{50}; WKY, 76.0 ± 6.2%; SHR, 61.0 ± 4.4%; P = 0.067).

**Immunohistochemistry**

PAR-2–like immunoreactivity (PAR-2-IR) was observed in both the endothelial and smooth muscle cells of the basilar artery but was more pronounced in the latter cell type (Figure 6). In both cell types, PAR-2-IR was observed on the cell surface as well as intracellularly. Smooth muscle cells exhibited more intense PAR-2-IR, which could be observed with higher titers of the primary antiserum. Smooth muscle cells also showed considerable variation in staining, ranging from apparent absence of PAR-2-IR in some cells to complete
staining of the cytoplasm. Endothelial cells, in contrast, shared consistently patchy PAR-2-IR on the cell surface and intracellularly. We could not discern any difference in the intensity or distribution of PAR-2-IR between the 2 strains of rat. Preabsorption of the antiserum with the immunizing peptide completely prevented staining, suggesting that the observed immunofluorescence was specific for the C-terminus of PAR-2.

**Discussion**

The present results suggest that, unlike other endothelium-dependent vasodilators in the basilar artery, vasodilator responses to PAR-2 activation are not impaired in the SHR model of chronic hypertension. Furthermore, responses to PAR-2 activation in both normotensive and hypertensive rats were entirely dependent on the synthesis of NO. Hence, although endothelial production of NO has been shown to be impaired in this model of hypertension, selective upregulation or preservation of the PAR-2–dependent mechanism, rather than recruitment of alternative endothelium-derived dilator substances (such as endothelium-derived hyperpolarizing factor), appears to maintain cerebral vasodilator responsiveness to PAR-2 activation. Therefore, our results point to a potential protective role for PAR-2 in cerebrovascular pathophysiology.

**Mechanism of PAR-2 Vasodilatation**

Previous studies have indicated that activation of PAR-2 produces endothelium-dependent, NO-mediated relaxation of isolated peripheral arteries. We reported recently that topical application of the rat PAR-2 synthetic tethered ligand sequence, SLIGRL-NH₂, to the basilar artery in vivo produced dilatation in Sprague-Dawley (normotensive) rats that was blocked by inhibitors of NO synthase or soluble guanylate cyclase. Thus, we concluded that, like acetylcholine, PAR-2 dilator responses of the basilar artery were also mediated by endothelial cell production of NO, which was confirmed in the present study in the WKY strain of rat. In addition, we found that PAR-2-IR was present in endothelial cells of the basilar artery. Thus, our studies provide evidence that PAR-2 activation (by SLIGRL-NH₂ and trypsin) causes endothelium-dependent dilatation of cerebral arteries by stimulating the release of NO. Therefore, if this efficacious NO-dependent response to PAR-2 activation is endothelium dependent, then the relaxant potency of

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**Figure 5.** Endothelium-dependent relaxation to SLIGRL-NH₂, trypsin, and acetylcholine of 5-HT–precontracted basilar artery rings from WKY and SHR. a, The relaxant effect of SLIGRL-NH₂ was ~3-fold more potent in SHR (n=5) than WKY (n=6). Relaxant effects of trypsin (b) (WKY, n=6; SHR, n=6) and acetylcholine (c) (WKY, n=5; SHR, n=7) were not significantly different between rat strains. pEC₅₀ values from each experiment are shown at the top right of each graph. Force of precontractions by 5-HT (10⁻⁸ to 10⁻⁷ mol/L) in each group were as follows: (a) WKY=5.3±1.1 mN, SHR=5.6±0.8 mN; (b) WKY=5.8±1.1 mN, SHR=5.8±1.0 mN; (c) WKY=7.9±1.7 mN, SHR=8.0±2.2 mN. *P<0.05, WKY vs SHR.

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**Figure 6.** Photomicrographs showing PAR-2–like immunoreactivity in the basilar artery of WKY (a) and SHR (b). PAR-2 was localized in both endothelial and vascular smooth muscle cells, as revealed by indirect fluorescence immunohistochemistry in both rat strains. Note the faint and patchy staining of endothelial cells (arrowheads) compared with the underlying smooth muscle cells. Also note the variation in intensity of fluorescence among different smooth muscle cells. Bar=25 μm.
PAR-2 receptors on the cerebral artery endothelium as determined in the present study by PAR-2 immunohistochemistry suggests that these relatively sparse receptors are either very well coupled to NO synthase or perhaps not involved in the response at all. If the latter was the case, then it is possible that the abundantly expressed smooth muscle PAR-2 is indirectly coupled to endothelial NO synthase. Since there is evidence to support the concept that a signal originating in vascular smooth muscle cells can act on the endothelium to cause synthesis of NO, it remains possible that smooth muscle PAR-2 contributes to the pronounced endothelium-dependent responses to PAR-2 agonists observed in this study.

Both our previous and present data indicate that, unlike SLIGRL-NH$_2$, trypsin is a poor vasodilator when applied topically to cerebral arteries in vivo. By contrast, we found here that low concentrations of both SLIGRL-NH$_2$ and trypsin produced near-complete relaxation of isolated basilar artery rings in which the endothelium was directly accessible. An explanation for this apparent discrepancy may be poor diffusion of trypsin to the endothelium in vivo such that effective concentrations of the enzyme were not reached to cause receptor activation. Another possibility is that endogenous antiproteases are present throughout the arterial wall in vivo but not in vitro. This latter possibility or an analogous mechanism would need to be invoked to explain the lack of effect of trypsin if, as hypothesized above, smooth muscle cell PAR-2 causes indirect activation of endothelial cell NO synthase.

Although PAR-2 mRNA and protein have been localized in vascular smooth muscle, activation of PAR-2 in the absence of endothelial factors generally has no effect on smooth muscle tone. However, in a recent study we showed that smooth muscle PAR-2 mediates contraction of the mouse renal artery. Although we observed abundant PAR-2-IR in the media of the basilar artery, unlike our previous study, their activation after inhibition of NO synthase failed to cause any direct contractions. Thus, as in many other arteries, smooth muscle PAR-2 does not appear to mediate any direct contractile effects on the basilar artery. The apparent variation in PAR-2-IR between individual smooth muscle cells observed here in our preliminary histochemical studies may indicate that PAR-2 mediates mitogenic effects involved in the growth and structural maintenance of the vascular wall rather than causing direct effects on smooth muscle tone.

Prolonged exposure (for up to 20 minutes) of isolated arteries to SLIGRL-NH$_2$ or trypsin has been shown to produce homologous desensitization of PAR-2, thus inhibiting responses to subsequent applications of either agent. From our studies, however, there was no evidence for desensitization of PAR-2 in vivo, because cumulative applications of 3 concentrations of SLIGRL-NH$_2$ (10$^{-6}$, 3×10$^{-6}$, and 10$^{-5}$ mol/L, applied for 5 minutes each) produced vasodilator responses that were fully reproducible in both WKY and SHR within 60 minutes. Therefore, as in our previous study, it is unlikely that desensitization contributed to the attenuation of SLIGRL-NH$_2$ responses after treatment with L-NNA.

### Effects of Chronic Hypertension on Dilator Responses of the Basilar Artery

In comparison to responses in WKY, dilator responses of the basilar artery in vivo to acetylcholine were markedly impaired in SHR. By contrast, vasodilator responses to sodium nitroprusside were preserved in SHR. These findings are consistent with previous reports that vasodilator responses of the basilar artery to endothelium-dependent agonists such as acetylcholine and bradykinin are selectively impaired in vivo during chronic hypertension and are not related to the smaller diameter of the basilar artery in SHR.

Moreover, our data suggest that the maximum vasodilator response to SLIGRL-NH$_2$ is selectively preserved in SHR in vivo, in contrast to that for acetylcholine, which is reduced by $\approx 65\%$ in SHR versus WKY. Interestingly, we found that relaxant responses to acetylcholine were not as severely impaired in the isolated basilar artery of SHR. Others have reported similar minimal effects of chronic hypertension on endothelium-dependent relaxation of the rat isolated basilar artery to acetylcholine and bradykinin. One reason for the difference in the effect of hypertension on endothelium-dependent relaxation between in vitro and in vivo assays might be related to different mechanisms underlying vascular tone in vivo and that induced by exogenous addition of vasoconstrictors in vitro as well as different mechanical forces that act on the vessel wall in each assay. Regardless of the reasons why the relaxations to acetylcholine appeared to be more affected by hypertension in vivo assay conditions compared with those in vitro, the clear increase in sensitivity to SLIGRL-NH$_2$ in the SHR strain under in vitro conditions provides additional support for our claim from the in vivo data that PAR-2–mediated cerebral vasodilatation is selectively preserved or even augmented after development of chronic hypertension.

Impaired dilator responses of the basilar artery to acetylcholine in vivo can be restored by the addition of L-arginine. This suggests that the impairment of endothelium-dependent basilar artery dilatation during chronic hypertension may be due to depletion or insufficient bioavailability of L-arginine rather than production of endothelium-derived contractile factors. Our findings, however, indicate that unlike acetylcholine and bradykinin, cerebral vasodilatation to PAR-2 activation is protected from such effects of chronic hypertension. Normal levels of NO may therefore be produced by endothelial cells in response to PAR-2 activators despite reduced basal levels of enzyme substrate in vivo. Since, in our experience, activation of PAR-2 elicits the most effective endothelium-dependent dilatation of this artery in vivo (eg, compared with acetylcholine and bradykinin), it is possible that the degree of endothelial dysfunction present in SHR is insufficient to blunt responses to PAR-2 activation, whereas responses to “poorer” vasodilators are more susceptible to hypertension.

As for endothelium-dependent relaxation to acetylcholine and bradykinin in vitro, relaxation to trypsin tended to be reduced by chronic hypertension. Therefore, chronic hypertension appears to selectively sensitize cerebral artery endothelial cell PAR-2 to the exogenous ligand, while, like acetylcholine, it reduces that to trypsin. As mentioned above,
the ineffectiveness of trypsin as a PAR-2 activator in vivo could also be due to the presence of antitrypsins in the vessel wall. Thus, retention of some of these inhibitors in vitro could also help explain the apparently diminished response to trypsin. Another explanation for the increased sensitivity to SLIGRL-NH$_2$ in chronic hypertension both in vitro and in vivo is that peptidases, which break down or inactivate exogenously applied peptide, may be downregulated in SHR. This could then allow a higher degree of bioavailability of the exogenous peptide for the receptor. Another possibility is that the accessibility of SLIGRL-NH$_2$ to the binding site on extracellular loop 2 of PAR-2 may be enhanced in SHR, while that for the tethered ligand exposed by trypsin is unaltered or even reduced. A more plausible explanation for the selective increase in sensitivity to SLIGRL-NH$_2$ in the SHR, however, may involve PAR-2 recycled back to the endothelial cell membrane in a “truncated” form after their enzymatic activation in vivo. After activation, both PAR-1 and PAR-2 are rapidly internalized into early endosomes and subsequently degraded within lysosomes. A small proportion of PARs avoid degradation and are returned to the plasmalemma without an N-terminal sequence, allowing activation only by synthetic tethered ligands. As such, our present observations could be explained if an increased number of PAR-2 exist in this truncated form during chronic hypertension. The relevance of such recycling of truncated PARs remains unknown; indeed, it may have no physiological role but rather may represent an “overloaded” lysosomal degradative pathway such that inappropriate turnover occurs.

Lack of Effect of PAR-1 Activators on Basilar Artery Tone

The lack of effect of thrombin and SFLLRN-NH$_2$, both in vivo and in vitro suggests that PAR-1 is not expressed in the rat basilar artery or, if it is, then it does not contribute to vascular tone under our experimental conditions. Furthermore, because thrombin also activates PAR-3 and PAR-4, it is unlikely that these receptors contributed to the regulation of vascular tone in the rat basilar artery. Thrombin has been reported to exert endothelium-dependent relaxations in isolated cerebral arteries from dogs and humans. In dogs the predominant response was a direct contraction, whereas only relaxation occurred in the human arteries. An important area of future research will be to characterize the PARs present in human cerebral vessels.

Physiological and Pathophysiological Roles of Vascular PARs

While our findings indicate that PAR-2 may be important in the regulation of cerebrovascular tone in vivo, identification of the endogenous activator(s) of PAR-2 and the effects that disease states may have on responses to PARs, as well as on expression and activity of the as yet unidentified regulators of PARs, remain unknown. While cloned and expressed PAR-2 has been shown to be activated by mast cell tryptase, two lines of evidence suggest that this tryptasinlike enzyme is not an activator of PAR-2 in the rat basilar artery. First, in contrast to peripheral vessels, where mast cells are commonly found in the adventitia, in cerebral vessels they are mainly restricted to the microvasculature of the dura and meninges. Similarly, in this study we could not detect any mast cells surrounding the basilar artery in the rat (J.D. Moffatt, PhD, unpublished data, 1998). The second reason why mast cell tryptase is unlikely to be an endogenous activator of cerebral PAR-2 is that application of trypsin to the adventitial side of the basilar artery in vivo appeared to have very limited access to endothelial PAR-2. If we assume that the NO-dependent relaxation to PAR-2 observed in this and our previous study is due to endothelial cell PAR-2 (and not to smooth muscle cell PAR-2; see above), then large PAR-2–activating molecules such as trypsin (molecular weight = 24 000) and tryptase (molecular weight = 35 000) are unlikely to be able to penetrate the medial layer of this artery to activate the endothelial cell receptors. Therefore, because of this diffusion “barrier” and the lack of mast cells within the artery wall, we propose that the endogenous activator of cerebral and perhaps most vascular PAR-2 originates from within the lumen of the artery. One possible candidate is the coagulation factor Xa, which has been shown to activate PAR-2 in isolated rat aorta.

An established feature of hypertension in humans and in experimental animal models is increased thrombotic potential. This phenomenon is associated with impaired endothelial release of anticoagulant substances such as NO and prostacyclin as well as a profile of plasma factors that are indicative of increased thrombogenesis. While endothelial dysfunction is generally diagnosed in terms of a poorer vasorelaxant responsiveness to endothelium-dependent vasodilator substances such as acetylcholine, its effects on coagulation have received considerably less attention. From our studies, however, we speculate that PAR-2–mediated NO production is preserved or even enhanced during the development of chronic hypertension and that such a mechanism serves to spare or retard the loss of the critical anticoagulant function of the cerebral vascular endothelium.

In summary, the present findings confirm that activation of PAR-2 is an effective and powerful vasodilator mechanism in cerebral arteries, which appears to involve production of endothelium-derived NO. Furthermore, whereas similar endothelium- and NO-mediated responses to other vasodilators such as acetylcholine and bradykinin are impaired during chronic hypertension, those to PAR-2 are largely preserved or augmented, which, we propose, is an indicator of important protective roles for PAR-2 against stroke in this vascular bed during hypertension.

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References


The vascular relaxation and consequent vasodilation from acetylcholine is depressed in chronic hypertension. This abnormality is also seen in SHR. The abnormalities are eliminated by the addition of arginine, suggesting that the depressed responses are due to depletion of arginine.

The accompanying article by Sobey and colleagues provides new information that may help to clarify the mechanisms of the depressed vascular response from acetylcholine in the basilar artery. These investigators found that responses to acetylcholine in the SHR were depressed, while responses due to activation of PAR-2 were intact. This result was surprising since responses to acetylcholine as well as to activation of PAR-2 are due to increased NO synthesis in endothelium. There is no clear explanation as to why the responses to one mechanism are preserved while the responses to the other are severely reduced, especially when one takes into account the evidence that abnormal responses to acetylcholine are due to depletion of arginine. A possible explanation is that acetylcholine is coupled to NO synthase that has no access to arginine, while the NO synthase coupled to PAR-2 does. This suggests that there is compartmentalization of the 2 mechanisms in endothelium. An additional finding that is difficult to explain is that the responses to acetylcholine in the basilar artery in vitro were largely preserved in contrast to what happens in the same vessel in vivo. Clearly additional information is needed to confirm these findings and to provide more definitive explanations for the surprising findings.

In addition to the bearing that these findings have on the mechanisms of the abnormal responses to acetylcholine in chronic hypertension, they do show that there is an additional mechanism for activation of endothelial NO synthase. The task for the future is to identify the physiological and pathophysiological mechanisms that activate this mechanism.

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Evidence for Selective Effects of Chronic Hypertension on Cerebral Artery Vasodilatation to Protease-Activated Receptor-2 Activation
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